

This Page Is Inserted by IFW Operations  
and is not a part of the Official Record

## **BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

**IMAGES ARE BEST AVAILABLE COPY.**

As rescanning documents *will not* correct images,  
please do not report the images to the  
**Image Problem Mailbox.**

# Rh(D) Antigen Expression and Isolation of a New Rh(D) cDNA Isoform in Human Erythroleukemic K562 Cells

By Kimita Suyama, Ruth Lunn, Stephanie Haller, and Jack Goldstein

Human erythroleukemic K562 cells are known to have several erythroid properties. K562 cells possess Rh mRNAs, but expression of Rh proteins has not previously been reported. We immunoprecipitated Rh protein from K562 cell lysate using rabbit anti-Rh and detected Rh(D) antigens on K562 cells using fluorescence-activated cell sorting (FACS). These results suggest that K562 cells will be useful as an expression model for most Rh antigens. We also cloned a new Rh(D) cDNA isoform (RhK562-II), from a K562 cDNA library using polymerase chain reaction (PCR) with 5' and 3' end oligonucleotides of the published Rh(e/E) antigen encoding cDNA sequence as primers. Sequence analysis showed that RhK562-II is composed of 951 nucleotides (316 amino acids), identical to the first 939 nucleotides (exons 1 to 6) of one of

the Rh(D) cDNAs (RhXIII), except for nucleotide 654 (C → G exchange). However, this exchange is the same as that of another published Rh(D) cDNA (RhPII cDNA). RhK562-II is deprived of exons 7 and 8 (nucleotides 940 to 1,153), followed by an identical sequence up to the 3' end of the open-reading frame of the RhXIII cDNA, which causes a frameshift mutation and produces a premature stop codon. In vitro expression of RhK562-II using the transcription and translation rabbit reticulocyte lysate system produced two major Rh-related proteins (30 kD and 25 kD), which were immunoprecipitated by rabbit polyclonal anti-Rh and separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

© 1994 by The American Society of Hematology.

**T**HE RH BLOOD GROUP antigens, especially Rh(D), are of paramount importance in transfusion medicine, ranking second only to the ABO system. These antigens (C/c, D, E/e) are carried by distinct but highly homologous unglycosylated, fatty-acylated proteins that share an identical N-terminal amino acid sequence.<sup>1-5</sup> Rh antigen expression occurs early in hematopoietic differentiation, being detected in colony-forming unit-erythroid (CFU-E) cells, but not burst-forming unit-erythroid (BFU-E) cells.<sup>6</sup> However, they have not been detected in human erythroleukemic K562 cells, which are at the proerythroid stage of erythroid development, even though Rh mRNA is present.<sup>7</sup>

Further elucidation of the structure and antigenicity of these Rh proteins can be facilitated by molecular analysis. To date, the genes encoding several Rh antigens and an Rh-related glycosylated protein that might be important in Rh antigenicity have been cloned. Two research groups have independently isolated and sequenced a cDNA gene from a human bone marrow library specific for the Rh(E) or (e) antigen.<sup>7,8</sup> The predicted translated product of this gene is a 417-amino acid protein with a molecular weight of 45.5 kD; however, this protein migrates anomalously on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (33 kD) due to its high hydrophobicity.<sup>9</sup> Other reported Rh cDNA clones include Rh(D) from a human bone marrow library<sup>10</sup> and two Rh-related clones from cultured erythroid cells.<sup>11</sup> We recently reported transient expression of a Rh cDNA clone in COS-1 cells.<sup>12</sup>

Genomic studies by Colin et al<sup>13</sup> support the two-locus model of Rh inheritance. According to this model, Rh-positive individuals have two Rh polypeptide genes, whereas only one gene is found in Rh-negative individuals, which suggests that one gene encodes for Rh(D) and the other Rh(C/c) and Rh(E/e). Although there appear to be only two Rh genes, the Rh blood group system is extremely polymorphic. The discovery of several Rh mRNAs from a human bone marrow library that are derived from alternative splicing of the gene encoding C/c and E/e provides an explanation of Rh diversity from one gene.<sup>14</sup>

We describe here the isolation of a new Rh cDNA clone from a human erythroleukemic K562 library, which is a

splicing isoform of the gene encoding the Rh(D) antigen. In addition, we report the expression of Rh polypeptides in K562 cells. Thus, K562 cells may provide a model system to study Rh antigenicity, since both polymorphisms and protein expression can be studied. Following submission of this report, a report has appeared that describes two Rh(D) variants associated with red blood cells.<sup>15</sup>

## MATERIALS AND METHODS

**Materials.** Human erythroleukemic K562 cells were purchased from American Type Culture Collection (Rockville, MD), and K562 cDNA library from Clontech Laboratory (Palo Alto, CA). Restriction enzymes (*Eco*RI and *Bam*HI) were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN), LambdaSorb phage adsorbent from Promega (Madison, WI), and L-<sup>35</sup>S-methionine, <sup>32</sup>P-labeled adenosine 5'-triphosphate, tetra (triethylammonium) salt was purchased from Dupont-NEN (Boston, MA). The sequenase 2.0 kit was obtained from USB, Cleveland, OH, and all cell culture media from Gibco-BRL, Grand Island, NY. Phenylmethylsulfonyl fluoride (PMSF), Trasylol, Protein A-Sepharose, N-tosyl-L-phenylalanine chloromethyl ketone (TPCK), and fluorescein isothiocyanate (FITC)-labeled goat antihuman Ig were obtained from Sigma (St Louis, MO).

**Fluorescence-activated cell sorting (FACS).** Human erythroleukemic K562 cells (10<sup>6</sup> cells) were incubated with 0.2 mL polyclonal human anti-D (dilution 1:10) for 30 minutes at 4°C and washed three times in phosphate-buffered saline (PBS) with 0.1% NaN<sub>3</sub> and 0.5% bovine serum albumin (BSA). The cells were reincubated with FITC-labeled goat antihuman Ig for 30 minutes at 4°C, washed three times,

---

From the Lindsley F. Kimball Research Institute, New York Blood Center, New York, NY.

Submitted October 22, 1993; accepted May 11, 1994.

Supported by Office of Naval Research Grant No. N00014-93-1-0364.

Address reprint requests to Jack Goldstein, PhD, New York Blood Center, 310 E 67 St, New York, NY 10021.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1994 by The American Society of Hematology.

0006-4971/94/8406-0009\$3.00/0

and resuspended in PBS with 0.1%  $\text{NaN}_3$  and 0.5% BSA. The cells were analyzed by FACS IV (Becton Dickinson, Sunnyville, CA). Data are presented as fluorescence intensity in fluorescence units versus cell frequency.

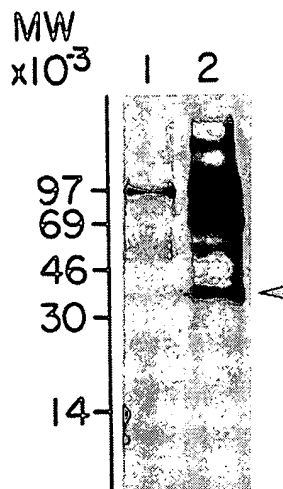
**Immunoprecipitation of Rh proteins from K562 cells with rabbit polyclonal anti-Rh.** Logarithmically growing K562 cells in complete RPMI media were collected and resuspended in RPMI without methionine. The cells ( $4 \times 10^7$  cells) were incubated with  $^{35}\text{S}$ -methionine (200  $\mu\text{Ci/mL}$ ) in 4 mL of the above media for 2 hours at  $37^\circ\text{C}$ . After washing three times with PBS, the cells were lysed by incubating with lysis buffer (0.25% SDS, 1% Triton X-100, 1 mmol/L TPCK, 1 mmol/L PMSF, and 100 U/mL Trasylol in 20 mmol/L Tris, pH 7.4) for 15 minutes at  $4^\circ\text{C}$ , and divided into four 1-mL aliquots. Supernatants collected by spinning were incubated with rabbit polyclonal anti-Rh (1:200 dilution) overnight at  $4^\circ\text{C}$ . Immune complexes extracted by Protein A-Sepharose were run on SDS-PAGE, followed by autoradiography.

**Cloning of Rh cDNA by polymerase chain reaction (PCR).** Preparation of template DNA from a human erythroleukemic K562  $\lambda\text{gt}11$  cDNA library was performed using the LambdaSorb phage adsorbent method. As primers, a 5' end oligonucleotide with initiation codon (sense primer, 5'-GGATGAGCTCTAAGTACCCG-3') sequence identical to that published for the Rh(e/E) cDNA clone, and a 3' end oligonucleotide with a stop codon (antisense primer, 5'-CCCGATCCTTAAATCCAACAGCCAA-3'), were synthesized at the Microchemistry Laboratory of the New York Blood Center.

PCR amplification of DNA was performed using 0.05 mmol/L dNTP plus 0.75 mmol/L  $\text{MgCl}_2$  in 10 mmol/L Tris-HCl, pH 9.0, with 50 mmol/L KCl and 0.1% Triton X-100. The reaction was performed in a MiniCycler (MJ Research, Watertown, MA) for 36 cycles, with each cycle consisting of denaturation at  $94^\circ\text{C}$  for 1 minute, annealing at  $50^\circ\text{C}$  for 2 minutes, and chain extension at  $72^\circ\text{C}$  for 3 minutes. PCR products were analyzed on 1% agarose gel. Southern blot analysis of the isolated cDNA was performed using a  $^{32}\text{P}$ -labeled oligonucleotide (5'-GAGGGAACGGAGGAT-AAA-3') specific for nucleotide 577 to 595 of the clone Rh(e/E) cDNA. The entire nucleotide sequence of the isolated cDNA was determined by the dideoxy-chain termination method using several oligonucleotides of the cloned Rh(e/E) cDNA, after subcloning the cDNA in pCRII vector (USB).

**Expressions of Rh proteins in vitro.** Expression of Rh proteins in vitro was performed with transcription- and translation-coupled reticulocyte lysate systems (Promega) according to the manufacturer's protocol and using Rh cDNA subcloned into pCRII as the expression vector [pCRIIRh(D) and pCRIIRhK562-II]. The reaction mixtures were incubated at  $30^\circ\text{C}$  for 90 minutes. Expressed Rh proteins were then immunoprecipitated with rabbit polyclonal anti-Rh or anti-RhNt<sup>16</sup> by incubating overnight at  $4^\circ\text{C}$ . The immune complexes were incubated with Protein A-Sepharose for 2 hours at  $4^\circ\text{C}$ , washed three times with lysis buffer and one time with 150 mmol/L NaCl in 50 mmol/L Tris, pH 6.8, eluted by boiling for 5 minutes in 1% SDS plus 8 mol/L urea plus 5%  $\beta$ -mercaptoethanol in 0.16 mol/L Tris, pH 6.8, and run on SDS-PAGE (12%), followed by autoradiography.

**Determination of relative abundance of RhK562-I transcripts to native RhK562-II by reverse transcription coupled with PCR amplification.** Total RNA was extracted from K562 cells using a RNA separation kit (Clontech). Briefly, total RNAs (1  $\mu\text{g}$ ) were incubated with oligo (dT) 12-18 primers at  $70^\circ\text{C}$  for 2 minutes. One tenth of the synthesized cDNAs were subjected to PCR amplification under the conditions described previously. PCR products were separated on 0.7% agarose gel and then transferred to Hybond-N+ (Amersham, Arlington Heights, IL) for Southern blot analysis.  $^{32}\text{P}$ -labeled oligonucleotide (5'-GCAATCCTGCTGGACGGCTTC-3') specific for



**Fig 1.** Autoradiogram of immunoprecipitates obtained from metabolically labeled K562 cells with  $^{35}\text{S}$ -methionine using rabbit polyclonal anti-Rh. Details are described in the Methods. Lane 1, preimmune rabbit serum; lane 2, rabbit polyclonal anti-Rh. Arrow shows Rh protein region.

nucleotide 271 to 291 of the cloned Rh(D) cDNA was used as a probe. After hybridization at  $38^\circ\text{C}$  overnight, Hybond-N+ was washed with  $0.2\times$  SSC ( $1\times$  SSC = 0.15 mol/L NaCl + 0.015 mol/L sodium citrate) and 0.1% SDS for 30 minutes at  $48^\circ\text{C}$  as a final wash.

## RESULTS

**Rh antigen expression in human erythroleukemic K562 cells.** Human erythroleukemic K562 cells have many erythroid properties, including the expression of red blood cell membrane proteins, such as glycophorin A.<sup>17</sup> In fact, Cherif-Zahar et al<sup>7</sup> detected Rh mRNA in these cells; however, expression of Rh protein has not been reported. To investigate Rh protein expression in K562 cells, we reacted a lysate prepared from K562 cells metabolically labeled with  $^{35}\text{S}$ -methionine with a rabbit anti-Rh antibody. A Rh polypeptide band was found at the expected position (32 kDa), as well as high-molecular weight polypeptides on SDS-PAGE (Fig 1, lane 2), while nonimmunized rabbit serum did not react with the large majority of these polypeptides (Fig 1, lane 1). These results suggest that Rh protein is expressed in K562 cells. The higher-molecular weight polypeptides could represent aggregates of the 32-kDa Rh protein and/or Rh-specific or -nonspecific complexes. The Rh molecule is known to aggregate due to its extreme hydrophobicity. We have observed a similar pattern of high-molecular weight oligomers as depicted in Fig 1 in immunoprecipitates using our Rh-specific rabbit antibody from the lysates of COS-1 cells and rabbit reticulocytes transfected with Rh(D) cDNA (data not shown). Since neither of these nontransfected cells originally contain Rh proteins, the high-molecular coprecipitates cannot be due to Rh protein-glycoprotein complexes. On the other hand, immunoprecipitation of higher-molecular polypeptides with Rh-specific antibodies has been well documented. For example, Moore et al<sup>18</sup> reported the coprecipitation of 50-kDa and 68-kDa polypeptides, as well as the 32-kDa Rh protein, from  $^{125}\text{I}$ -labeled red blood cells using human anti-D plasma. Evidence for the existence of Rh protein-glycoprotein complexes in K562 cells is supported by the

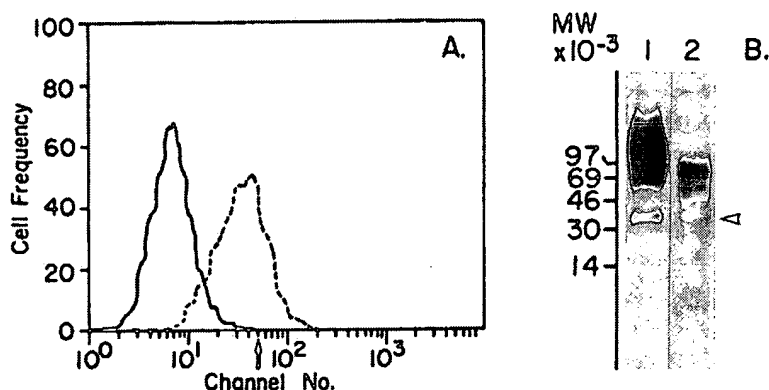


Fig 2. FACS profile of K562 cells in the presence of the human polyclonal anti-D and isolation of Rh(D) polypeptide from <sup>125</sup>I surface-labeled K562 cells by the same antibody. (A) K562 cells were incubated with a 1:10 dilution of either polyclonal anti-D or normal human type AB serum both containing 0.5% BSA and 0.1% NaN<sub>3</sub> in PBS. After washing, the cells were stained with FITC-conjugated goat antihuman Ig and analyzed by FACS. Details are described in the Methods. (—) Cells treated with control serum; (---) cells treated with human monoclonal anti-D. Arrow shows channel 50. (B) According to the method reported by Moore et al.,<sup>18</sup> human red blood cells (R<sub>1</sub>R<sub>2</sub>) and K562 cells were first surface-labeled with <sup>125</sup>I, then sensitized with human polyclonal anti-D and solubilized. The immune complexes bound to Protein A-Sepharose were eluted and run on SDS-PAGE (12%). Lane 1, human red blood cells; lane 2, K562 cells. Arrow shows Rh(D) polypeptides.

isolation of the Rh glycoprotein in these cells (unpublished data). While the nature of these Rh-specific high-molecular weight oligomers cannot as yet be clearly defined, it seems to be characteristic of Rh immunoprecipitations.

To ascertain if expression occurred on the cell surface at this stage of erythroid development, we sensitized K562 cells with a human polyclonal anti-D, followed by an incubation with a second antibody (FITC-labeled antihuman Ig). These cells were analyzed by FACS (Fig 2A). K562 cells sensitized with anti-D exhibited a significant fluorescence intensity (channels 50 through 100) not observed with nonsensitized control K562 cells (channels 1 through 50) or with cells sensitized with anti-Fy<sup>a</sup> Duffy, a blood group antigen not present on K562 cells (data not shown).<sup>19</sup> Fluorescence intensity was dependent on the concentration of anti-D in these cells, as well as in human red blood cells. Hence, K562 cells appear to express Rh(D) antigens on their surface.

While data shown in Fig 1 and Fig 2 demonstrate the presence of Rh polypeptides in K562 cell lysate and Rh(D) antigens on the cell surface, respectively, they do not definitively show that the polypeptides immunoprecipitated by rabbit anti-Rh are the same antigens detected on the cell surface by human anti-D. For this reason, we reacted <sup>125</sup>I surface-labeled K562 cells with human polyclonal anti-D. After solubilization, and extraction by Protein A-sepharose, we recovered a 32-kD band by SDS-PAGE (Fig 2B). These results clearly establish the presence of Rh(D) antigen-carrying proteins in K562 cells.

**PCR amplification of Rh cDNA.** We then set out to determine whether we could isolate cDNA clones for both the Rh acyl-proteins and the RhD50, which is a glycoprotein coprecipitated with human monoclonal anti-D, from a human erythroleukemic K562 library.<sup>1</sup> PCR amplification using 5' and 3' end-specific oligonucleotides for the RhD50 cDNA yielded a 1.2-kb clone whose sequence corresponded to that of the published RhD50 (data not shown). Two PCR prod-

ucts were obtained using Rh(e/E) or Rh IXb 5' and 3'-specific primers: an expected 1.32-kb clone (RhK562-I cDNA) and an unknown 1-kb clone (RhK562-II cDNA), which may be a truncated gene of RhIXb. These bands were isolated and then rerun independently on a 1% agarose gel (Fig 3). Both of these clones bound strongly to a <sup>32</sup>P-labeled internal Rh(D)-specific oligonucleotide in Southern blot analysis (data not shown), which suggests that they were Rh-related cDNAs. Nucleotide sequencing using the di-deoxy-chain termination method was implemented after sub-cloning each clone separately into PCRII vectors. The nucleotide sequence of RhK562-I (data not shown) was identical to the recently published Rh(D) cDNA clone (RhXIII) isolated from human bone marrow.<sup>10</sup>

**Characterization of RhK562-II.** RhK562-II was of great

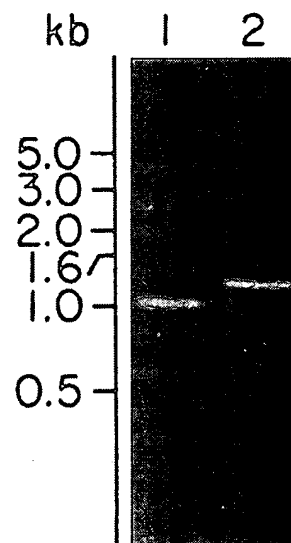


Fig 3. Cloning of a Rh cDNA from a human erythroleukemic cell (K562) cDNA library by PCR. Lanes 1 and 2, PCR products using 5' end and 3' end oligonucleotides of Rh cDNA as primers and K562 cell cDNA library as a template. Details are described in the Methods.

Met	Ser	Ser	Lys	Tyr	Pro	Arg	Ser	Val	Arg	Arg	Cys	Leu	Pro	Leu	Trp	Ala	Leu	Thr	Leu	20
ATG	AGC	TCT	AAG	TAC	CCG	CGG	TCT	GTC	CGG	CGC	TGC	CTG	CCC	CTC	TGG	GCC	CTA	ACA	CTG	60
Glu	Ala	Ala	Leu	Ile	Leu	Leu	Phe	Tyr	Phe	Phe	Thr	His	Tyr	Asp	Ala	Ser	Leu	Glu	Asp	40
GAA	GCA	GCT	CTC	ATT	CTC	CTC	TTC	TAT	TTT	TTT	ACC	CAC	TAT	GAC	GCT	TCC	TTA	GAG	GAT	120
Gln	Lys	Gly	Leu	Val	Ala	Ser	Tyr	Gln	Val	Gly	Gln	Asp	Leu	Thr	Val	Met	Ala	Ala	Ile	60
CAA	AAG	GGG	CTC	GTG	GCA	TCC	TAT	CAA	GTT	GGC	CAA	GAT	CTG	ACC	GTG	ATG	GCG	GCC	ATT	180
Gly	Leu	Gly	Phe	Leu	Thr	Ser	Ser	Phe	Arg	Arg	His	Ser	Trp	Ser	Ser	Val	Ala	Phe	Asn	80
GGC	TTG	GGC	TTC	CTC	ACC	TCG	AGT	TTC	CGG	AGA	CAC	AGC	TGG	AGC	AGT	GTG	GCC	TTC	AAC	240
Leu	Phe	Met	Leu	Ala	Leu	Gly	Val	Gln	Trp	Ala	Ile	Leu	Leu	Asp	Gly	Phe	Leu	Ser	Gln	100
CTC	TTC	ATG	CTG	GCG	CTT	GGT	GTG	CAG	TGG	GCA	ATC	CTG	CTG	GAC	GGC	TTC	CTG	AGC	CAG	300
Phe	Pro	Ser	Gly	Lys	Val	Val	Ile	Thr	Leu	Phe	Ser	Ile	Arg	Leu	Ala	Thr	Met	Ser	Ala	120
TTC	CCT	TCT	GGG	AAG	GTG	GTC	ATC	ACA	CTG	TTC	AGT	ATT	CGG	CTG	GCC	ACC	ATG	AGT	GCT	360
Leu	Ser	Val	Leu	Ile	Ser	Val	Asp	Ala	Val	Leu	Gly	Lys	Val	Asn	Leu	Ala	Gln	Leu	Val	140
TTG	TCG	GTG	CTG	ATC	TCA	GTG	GAT	GCT	GTC	TTG	GGG	AAG	GTC	AAC	TTG	GCG	CAG	TTG	GTG	420
Val	Met	Val	Leu	Val	Glu	Val	Thr	Ala	Leu	Gly	Asn	Leu	Arg	Met	Val	Ile	Ser	Asn	Ile	160
GTG	ATG	GTG	CTG	GTG	GAG	GTG	ACA	GCT	TTA	GGC	AAC	CTG	AGG	ATG	GTC	ATC	AGT	AAT	ATC	480
Phe	Asn	Thr	Asp	Tyr	His	Met	Asn	Met	Met	His	Ile	Tyr	Val	Phe	Ala	Ala	Tyr	Phe	Gly	180
TTC	AAC	ACA	GAC	TAC	CAC	ATG	AAC	ATG	GAC	ATC	TAC	GTG	TTC	GCA	GCC	TAT	TTT	GGG		540
Leu	Ser	Val	Ala	Trp	Cys	Leu	Pro	Lys	Pro	Leu	Pro	Glu	Gly	Thr	Glu	Asp	Lys	Asp	Gln	200
CTG	TCT	GTG	GCC	TGG	TGC	CTG	CCA	AAG	CCT	CTA	CCC	GAG	GGA	ACG	GAG	GAT	<u>AAA</u>	GAT	CAG	600
Thr	Ala	Thr	Ile	Pro	Ser	Leu	Ser	Ala	Met	Leu	Gly	Ala	Leu	Phe	Leu	Trp	<u>Met</u>	Phe	Trp	220
ACA	GCA	ACG	ATA	CCC	AGT	TTG	TCT	GCC	ATG	CTG	GGC	GCC	CTC	TTC	TTG	TGG	<u>ATG</u>	TTC	TGG	660
Pro	Ser	Phe	Asn	Ser	Ala	Leu	Leu	Arg	Ser	Pro	Ile	Glu	Arg	Lys	Asn	Ala	Val	Phe	Asn	240
CCA	AGT	TTC	AAC	TCT	GCT	CTG	CTG	AGA	AGT	CCA	ATC	GAA	AGG	AAG	AAT	GCC	GTG	TTC	AAC	720
Thr	Tyr	Tyr	Ala	Val	Ala	Val	Ser	Val	Val	Thr	Ala	Ile	Ser	Gly	Ser	Ser	Leu	Ala	His	260
ACC	TAC	TAT	GCT	GTA	GCA	GTC	AGC	GTG	GTG	ACA	GCC	ATC	TCA	GGG	TCA	TCC	TTG	GCT	CAC	780
Pro	Gln	Gly	Lys	Ile	Ser	Lys	Thr	Tyr	Val	His	Ser	Ala	Val	Leu	Ala	Gly	Gly	Val	Ala	280
CCC	CAA	GGG	AAG	ATC	AGC	AAG	ACT	TAT	GTG	CAC	AGT	GCG	GTG	TTG	GCA	GGA	GGC	GTG	GCT	840
Val	Gly	Thr	Ser	Cys	His	Leu	Ile	Pro	Ser	Pro	Trp	Leu	Ala	Met	Val	Leu	Gly	Leu	Val	300
GTG	GGT	ACC	TCG	TGT	CAC	CTG	ATC	CCT	TCT	CCG	TGG	CTT	GCC	ATG	GTG	CTG	GGT	CTT	GTG	900
Ala	Gly	Leu	Ile	Ser	Val	Gly	Ala	Lys	Tyr	Leu	Pro	Val	Cys	Ser	***					317
GCT	GGG	CTG	ATC	TCC	GTC	GGG	GGA	GCC	AAG	TAC	CTG	CCG	GTT	TGC	TCC	TAA				951

Fig. 4. DNA and the predicted protein sequence of RhK562-II cDNA. Box indicates the amino acid difference of the Rh(D) protein as a result of a single nucleotide difference (underlined).

interest to us because of its potential as a new Rh(D) cDNA-splicing isoform. Previously isolated truncated Rh clones have turned out to be Rh-splicing isoforms of the Rh(Cc/Ee)-specific gene.<sup>13</sup> Moreover, RhK562-I (which was isolated in the same PCR reaction as RhK562-II) was identical to Rh(D) cDNA; hence, there was a strong possibility that RhK562-II was an Rh(D) isoform. Thus, we characterized RhK562-II with respect to sequence analysis, membrane topology, and in vitro translation.

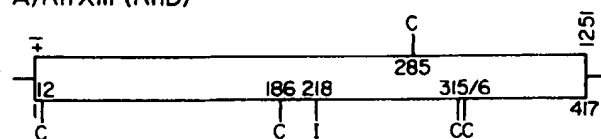
**Sequence analysis of RhK562-II.** Figure 4 depicts the complete nucleotide sequence of the 948-base open-reading frame clone, as well as the predicted 316 amino acids. Nucleotides 1 to 939 and 1,154 to 1,251 (data not shown) are identical to that of RhXIII, except for a C → G nucleotide exchange at position 654; however, this exchange may not be due to PCR error, since it is the same as another reported Rh cDNA clone.<sup>20</sup> A deletion occurs from nucleotides 950 to 1,153, causing a translation frame-shift that produces a premature stop codon at nucleotide 949 (Fig 5). Furthermore, this deletion corresponds to the reported intron/exon transitions, such that it encompasses exons 7 and 8. RhK562-II is an Rh isoform formed by alternative splicing of the Rh(D)-specific gene.

**Membrane topology of RhK562-II.** Figure 6 compares the membrane topology of the isoform RhK562-II based on the hydropathy analysis reported by Kyte et al<sup>21</sup> using a 6-amino acid window to that of the published prototype RhXIII.<sup>11</sup> The RhK562-II protein contains 10 transmembrane domains with both internal N- and C-termini, whereas the

RhXIII protein has 13 transmembrane domains with an intracellular N-terminus. Although the carboxy end of RhXIII is predicted to be exofacial, biochemical studies have yielded contrasting results.<sup>22</sup>

With respect to possible epitope considerations in RhK562-II the following are of interest: (1) the exofacial cysteine (285) thought to be critical to Rh antigenicity is

#### A) Rh XIII (RhD)



#### B) Rh K562-II

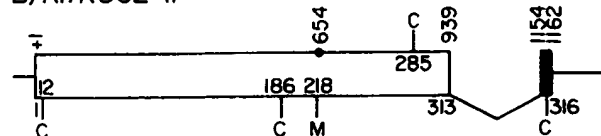


Fig 5. Comparison of RhK562-II to RhXIII (RhD) in nucleotide sequence and amino acid sequence. Open boxes represent coding sequences, and single lines indicate noncoding regions. Closed box indicates region of RhK562-II-encoding amino acids produced by frame-shift of RhXIII. Numbers below and inside boxes refer to amino acid positions starting from initiating methionine. Numbers above boxes indicate nucleotide positions starting from initiation codon. C, cysteine; M, methionine; I, isoleucine. Closed dot indicates the nucleotide difference (C → G).

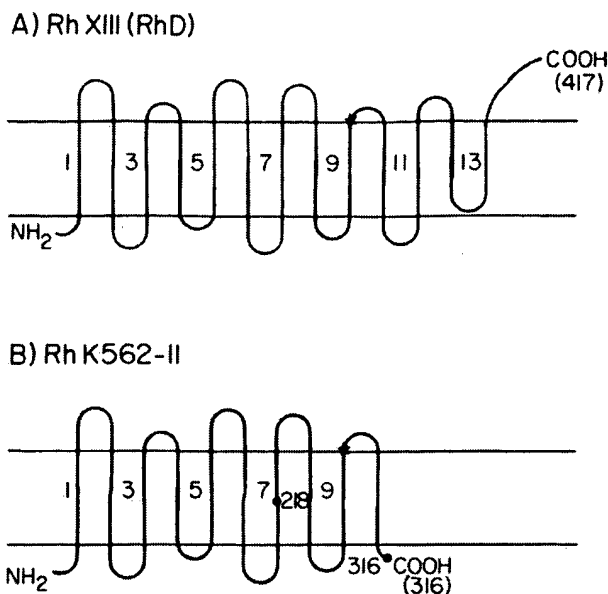


Fig 6. Comparison of membrane topology of the RhK562-II protein with that of RhXIII (RhD) protein. Membrane topology was predicted by hydropathy analysis according to Kyte et al<sup>21</sup> using a 6-amino acid window. Closed circles indicate amino acid polymorphisms. Stars indicate cysteine residue at 285.

conserved; (2) the N-terminus region containing a 17-kD chymotrypsin polypeptide, which is believed to be involved in Rh(D) antigen expression<sup>16</sup> is also conserved; and (3) the generation of a different C-terminus end due to the translational frame-shift mutation is unlikely to result in a new epitope, since it is intracellular and is only three amino acids long.

*In vitro translation of RhK562-II.* To ascertain if RhK562-II produced a viable protein recognized by Rh antibodies, we expressed this gene, as well as RhXIII, in a TNT transcription/translation-coupled reticulocyte lysate system. The expression vectors used in this system (pCRIIRhXIII and pCRIIRhK562-II) were constructed by subcloning each gene separately into pCRII vectors, which contain a T7 promoter. Rh antibodies used in the characterization of these translated proteins were a polyclonal rabbit anti-Rh that specifically binds to all Rh proteins, and a polyclonal rabbit anti-RhNt that recognizes the shared N-terminus of all Rh proteins. As shown in Fig 7, rabbit anti-Rh immunoprecipitated a 32-kD <sup>35</sup>S-methionine-incorporated protein produced by pCRIIRhXIII and two labeled proteins, an expected 30-kD, and a 26-kD produced by pCRK562-II. However, only the 30-kD band (Fig 7B) of the pCRIIRhK562-II translation products was immunoprecipitated with rabbit anti-RhNt. The smaller protein (26 kD) may be a product of a different initiation codon or a protease cleavage product in the N-terminal region.

We were interested in the expression of RhK562-II mRNA and the translated protein in K562 cells. As shown in Fig 1, a shortened Rh(D) polypeptide was not detected by immunoprecipitation using rabbit anti-Rh antibody. However, RhK562-II may exist as highly aggregated molecules and/

or may exhibit abnormal behavior on SDS-PAGE due to its high hydrophobicity. This is supported by a report from Mouro et al<sup>15</sup> that Rh proteins from D<sup>VI</sup>-type red blood cells, which lack exons 4, 5, and 6 of the Rh(D) gene, migrated as an unknown 69-kD protein, as well as 32-kD protein, on SDS-PAGE when tested by Western blot analysis using a rabbit antibody against the C-terminal peptide of Rh(D) proteins. However, we were able to determine the relative transcript abundance of RhK562-II to native RhK562-I in K562 cells using reverse-transcriptase PCR. Total RNAs were isolated from K562 cells, then cDNAs were synthesized from the RNAs by reverse transcriptase and the produced Rh(D) cDNAs were amplified by PCR using 5' and 3' end-specific primers of Rh(D) cDNA. PCR products were separated on a 0.7% agarose gel and transferred to Hybond-nylon. By Southern analysis, two bands, a 1.2-kb native RhK562-I and a 1.0-kb truncated RhK562-II, were detected using a nucleotide probe common to both native and truncated cDNAs (Fig 8). Relative abundance of RhK562-II to native RhK562-I was approximately 1:10.

## DISCUSSION

Human erythroleukemic K562 cells may represent an alternative model for the study of Rh antigenicity, not only in terms of Rh expression in hematopoietic differentiation, but also for investigation of Rh(D) complex formation and potential function of Rh proteins and polymorphism of the Rh antigen system. This report of Rh(D) protein expression and the isolation of a new Rh(D) isoform in K562 cells provides credibility for this model.

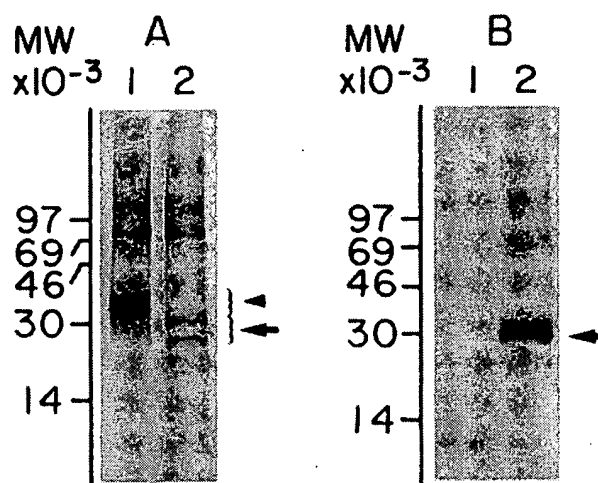


Fig 7. Expression of Rh cDNAs in a transcription/translation-coupled reticulocyte lysate system. (A) Autoradiogram of expressed Rh proteins obtained by immunoprecipitation with rabbit anti-Rh and separation on 12% SDS-PAGE. Lane 1, pCRIIRhXIII vector expression; lane 2, pCRIIRhK562-II vector expression. (B) Autoradiogram of expressed Rh proteins obtained by immunoprecipitation with rabbit anti-RhNt and separation on 12% SDS-PAGE. Lane 1, pCRII vector alone; lane 2, pCRIIRhK562-II vector. Details are described in the Methods. Arrowhead indicates Rh(D) protein region and arrow the RhK562-II protein region.

Kb

2.0 →  
1.2 →  
1.0 →  
0.5 →



**Fig 8. Relative abundance of RhK562-II transcripts to RhK562-I.** Total RNAs were isolated from K562 cells and the cDNAs were produced by reverse transcriptase using the total RNAs, then RhK562-I cDNA and RhK562-II cDNA were specifically amplified by PCR using 5' end and 3' end primers of Rh(D) cDNA. They were separated on a 0.7% agarose gel and detected by Southern blot analysis using a probe common to both. Details are described in the Methods.

Although Rh mRNA has been detected in K562 cells, protein expression has not been reported.<sup>7</sup> Possible reasons for undetectable Rh antigens are that K562 cells simply produce considerably fewer antigen sites per cell and/or may have a slightly different configuration of Rh antigens on their cell surfaces, compared with red blood cells, and possibly insufficient titers of anti-Rh antibodies were used for detection. The former explanation is supported by the findings of Cherif-Zahar et al<sup>7</sup> that Rh mRNAs are present in lower amount in K562 cells compared with adult spleen erythroblasts. However, K562 cells clearly produced Rh antigen (Figs 1 and 2A and B). Our isolation of the RhD50 protein provides further evidence that K562 cells have some of the necessary Rh-related polypeptides thought to be required for Rh antigen expression.

K562 cells may also represent a means to study the generation of Rh polymorphism. Rh is the most polymorphic of the 19 known red blood cell group systems, consisting of at least 45 reported antigens.<sup>23</sup> Variations in Rh(D) expression can be both qualitative (partial D red blood cells) and quantitative (weak D red blood cells).<sup>24</sup> Qualitative variation in D is believed to be due to differences in expression of the number and type of the eight postulated overlapping D epitopes.

Possible mechanisms for the generation of these Rh(D) polymorphisms include mutations and alternative splicing. Since the epitopes appear to be overlapping, a single mutation could account for loss of several epitopes. Alternative splicing has been found to be a means to generate diversity in glycoproteins C and D, which carry the Ge blood group system,<sup>23</sup> and Rh(C/c, E/e)-specific splicing isoforms have been isolated.<sup>13</sup> This report of a newly isolated Rh(D) isoform supports the model of alternative splicing as a significant mechanism of Rh diversity. Although this isoform was isolated from a human erythroleukemic K562 library, it is unlikely that it is merely the result of alternative expression that is developmentally regulated, since the prototype RhK562-I was also isolated from this same library. More-

over, three of the Rh(Cc/Ee) isoforms have been isolated from both human bone marrow and reticulocytes. Thus, we suspect that RhK562-II exists at different developmental stages. Epitope characterization of the expressed RhK562-II polypeptide will provide insight on how antigen diversity correlates with molecular RNA splicing.

#### ACKNOWLEDGMENT

We thank Jay Valinsky for his helpful advice, Harold Ralph for performing the FACS, and Tellervo Huima for her excellent photography.

#### REFERENCES

1. Agre P, Cartron JP: Molecular biology of Rh antigens. *Blood* 78:551, 1991
2. Hartel-Schenk S, Agre P: Mammalian red cell membrane Rh polypeptides are selectively palmitoylated subunits of a macromolecular complex. *J Biol Chem* 267:5569, 1992
3. Moore S, Woodrow CF, McClelland DBL: Isolation of membrane components associated with human red cell antigens Rh(D), (c), (E) and Fy<sup>a</sup>. *Nature* 295:529, 1982
4. Gahmberg CG: Molecular identification of the human Rh(D) antigens. *FEBS Lett* 40:93, 1982
5. Blanchard D, Bloy C, Hermand P, Cartron JP, Saboori AM, Smith BL, Agre P: Two-dimensional iodo-peptide mapping demonstrates that erythrocyte RhD, c and E polypeptides are structurally homologous but nonidentical. *Blood* 72:1424, 1988
6. Falkenburg FJH, Fibbe WE, van der Vaart-Duinkerken N, Nichols ME, Rubinstein P, Jansen J: Human erythroid progenitor cells express rhesus antigens. *Blood* 66:660, 1985
7. Cherif-Zahar B, Bloy C, Le Van Kim C, Blanchard D, Bailly P, Hermand P, Salmon C, Cartron JP, Colin Y: Molecular cloning and protein structure of a human blood group Rh polypeptide. *Proc Natl Acad Sci USA* 87:6243, 1990
8. Avent ND, Ridgwell K, Tanner MJA, Anstee DJ: cDNA cloning of a 30 kDa erythrocyte membrane protein associated with Rh (rhesus)-blood-group-antigen expression. *Biochem J* 271:821, 1990
9. Suyama K, Goldstein J, Aebersold R, Kent S: Regarding the size of Rh proteins. *Blood* 72:411, 1991
10. Le Van Kim C, Mouro I, Cherif-Zahar B, Raynal V, Cherrier C, Cartron JP, Colin Y: Molecular cloning and primary structure of the human blood group RhD polypeptide. *Proc Natl Acad Sci USA* 89:10925, 1992
11. Kajii E, Umenishi F, Iwamoto S, Ikemoto S: Isolation of a new cDNA clone encoding an Rh polypeptide associated with the Rh blood group system. *Hum Genet* 91:157, 1993
12. Suyama K, Roy S, Lunn R, Goldstein J: Expression of the 32-kd polypeptide of the Rh antigen. *Blood* 82:1006, 1993
13. Colin Y, Cherif-Zahar B, Le Van Kim C, Raynal V, Van Huffel V, Cartron JP: Genetic basis of the RhD-positive and RhD-negative blood group polymorphism as determined by Southern analysis. *Blood* 78:2747, 1992
14. Le Van Kim C, Cherif-Zahar B, Raynal V, Mouro I, Lopez M, Cartron JP, Colin Y: Multiple Rh messenger RNA isoforms are produced by alternative splicing. *Blood* 80:1074, 1992
15. Mouro I, Le Van Kim C, Rouillac C, van Rhenen DJ, LePenec PY, Bailly P, Cartron JP, Colin Y: Rearrangements of the blood group RhD gene associated with the DVI category phenotype. *Blood* 83:1129, 1994
16. Suyama K, Goldstein J: Membrane orientation of Rh(D) polypeptide and partial localization of its epitope-containing domain. *Blood* 78:808, 1992

17. Rutherford TR, Clegg JB, Weatherall DJ: K562 human leukemic cells synthesize embryonic haemoglobin in response to haemin. *Nature* 280:164, 1979
18. Moore S, Woodrow CF, McClelland DBL: Isolation of membrane components associated with human red cell antigens Rh(D), (c), (E) and Fy<sup>a</sup>. *Nature* 295:529, 1982
19. Chaudhuri A, Polyakova J, Zbrzezna V, Williams K, Gulati S, Pogo AO: Cloning of glycoprotein D cDNA, which encodes the major subunit of the Duffy blood group system and the receptor for the *Plasmodium vivax* malaria parasite. *Proc Natl Acad Sci USA* 90:10793, 1993
20. Arce MA, Thompson ES, Wagner S, Coyne KE, Ferdman BA, Lublin DM: Molecular cloning of RhD cDNA derived from a gene present in RhD-positive, but not RhD-negative individuals. *Blood* 82:651, 1993
21. Kyte J, Doolittle RF: A simple method for displaying the hydropathic character of a protein. *J Mol Biol* 157:105, 1982
22. Avent ND, Butcher SK, Liu W, Mawby WJ, Mallinson G, Parsons SF, Anstee DJ, Tanner MJA: Localization of the C termini of the Rh (rhesus) polypeptides to the cytoplasmic face of the human erythrocyte membrane. *J Biol Chem* 267:15134, 1992
23. Tippet P: Serologically defined Rh determinants. *J Immunogenet* 17:247, 1990
24. Issitt PD: The Rh blood group system, 1988: Eight new antigens in nine years and some observations on the biochemistry and genetics of the system. *Transfusion Med Rev* 3:1, 1989